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# Reverse transcriptase inhibits Taq polymerase activity

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## ABSTRACT

**Detection of viral RNA by polymerase chain reaction (PCR) requires the prior reverse transcription of the viral RNA. In order to minimise the number of manual manipulations required for processing large numbers of samples, we attempted to design a system whereby all the reagents required for both reverse transcription and amplification can be added to one tube and a single, non-interupted thermal cycling program performed. Whilst attempting to set up such a one-tube system with Taq polymerase (Taq; Biotech International) and avian myoblastosis virus (AMV) reverse transcriptase (RT), we noticed a sustantial decrease in the sensitivity of detection of viral RNA. Investigation of this phenomenon has revealed direct interference of RT with Taq polymerase. Evidence supporting this conclusion includes the following observations: (1) increasing the ratio of Taq to RT improves sensitivity; (2) adding non-homologous RNA improves sensitivity; (3) RT that has been heat inactivated prior to Taq addition does not exert this effect; (4) the effect is not sequence restricted; (5) the $Mg2+$ ions are not sequestered by RT. In addition, the effect is not limited to AMV RT, Moloney murine leukaemia virus RT also affects Taq activity.**

## INTRODUCTION

Since its first description by Saiki in 1985 (1), the polymerase chain reaction (PCR) has revolutionised the methodology of gene manipulation. The ability to amplify a specific region of DNA defined by a chosen set of oligonucleotide primers over a billion fold by repeated cycles of heat denaturation of DNA, annealing of the primers, and extension of the annealed primers by a DNA polymerase has had a significant impact on the area of nucleic acid detection. This effect has been most pronounced in the years since a thermostable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus* was applied to PCR in 1988 (2).

One area where PCR has shown great value is the detection of viral pathogens, particularly those for which culture is difficult or serologically based detection systems are inadequate. PCR has the advantages of being faster, more sensitive and more specific. It has been used successfully to detect a range of viruses , including both DNA viruses (e.g. Hepatitis B virus, papillomavirus) and RNA viruses (e.g. rotavirus, measles, influenza, Dengue and HIV-1), although RNA viruses must first be reverse transcribed to cDNA, since PCR can only amplify DNA sequences (3–10).

We intend to apply PCR to detect Ross River virus (RRV) in mosquitoes. RRV is a mosquito borne alphavirus that is the etiological agent of epidemic polyarthritis. A surveillance program currently exists in which mosquitos are trapped and assessed for virus carriage by tissue culture techniques. This process is time consuming, taking up to two weeks for virus isolation and identification.

PCR can potentially reduce this time, however due to the large quantities of mosquitos to be processed, the number of manual manipulations of samples for PCR must be minimised in order to be of net benefit. To this end, we attempted to devise a system by which the reverse transcription of the RRV RNA genome and amplification of a 549 bp fragment by PCR could occur in the same reaction tube in a single, non-interrupted thermal cycling program without the addition of extra reagents during the procedure.

Standard practice for assessing the value of PCR in viral detection is to determine the sensitivity level. This is achieved by testing serial dilutions of known virus load in the PCR system.

We initially found that reverse transcription followed by PCR (RT-PCR) in a one tube system was only successful if a relatively large amount of viral RNA was present. More interestingly, the positive control (plasmid DNA of a cDNA clone of the virus) failed to be amplified in the RT-PCR reaction, even though it was amplified successfully in the normal PCR reaction.

In this paper we describe an investigation of this phenomenon, which yielded the conclusion that reverse transcriptase can directly inhibit Taq polymerase activity, and the measures required to counter the inhibitory effect of reverse transcriptase.

## MATERIALS AND METHODS

### Enzymes

Taq polymerase was obtained from Biotech International Ltd., and supplied in a buffer containing 50% glycerol, 300 mM KCl, 1 mg/mL BSA, 0.4% Triton X-100, 1 mM $\beta$ mercaptoethanol, 1 mM EDTA, 20 mM Tris pH 7.6. Avian myeloblastosis virus (AMV) reverse transcriptase (RT) was obtained from Promega Corporation in buffer containing 50% glycerol, 100 mM $K_2PO_4$ pH 7.2, 0.2% Triton X-100, 2 mM dithiothreitol.

### Synthetic oligonucleotides

The sequences of the two RRV isolates in GenBank were compared on computer using the IBI Pustell DNA Sequence

Analysis programme to identify conserved regions, upon which the PCR primers were based. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer, deprotected and purified by Oligonucleotide Purification Cartridge (OPC, Applied Biosystems) treatment. The sequences of the two oligonucleotide primers are: RRV1449S; 5'-TCC GCC CAA ATA GGT CTG GA-3', RRV1979C; 5'-TGT CAT GGC TGG TAA CGG CA-3'. These hybridise to the E2 region of the viral genome, and yield a 549 bp product.

### Viral RNA preparation

Virus was propogated on Vero cells until cytopathic effect was evident (approximately 36 hours post-infection). Cell debris was removed by centrifugation and virus precipitated from the supernatant with 7% polyethyleneglycol 6000. Virus was collected by centrifugation, purified by sedimentation through a 10–40% sucrose gradient and concentrated by ultracentrifugation. Viral RNA was purified by Proteinase K treatment of virus, followed by two phenol chloroform extractions and ethanol precipitation.

### RT-PCR

Standard conditions for the reverse transcription of the viral RNA and amplification of the 549 bp sequence were as follows. Each reaction tube contained target viral RNA or plasmid DNA in various dilutions, 20 pmol of each primer, 100 $\mu$M of each of the four deoxynucleotide triphosphates (Pharmacia LKB Biotechnology, USA), 2 mM $MgCl_2$, 1×reaction buffer containing 67 mM Tris-HCl pH 8.8, 17 mM $(NH_4)_2SO_4$, 1 mM $\beta$ mercaptoethanol, 6$\mu$M EDTA, 0.2 mg/mL gelatin (Biotech International Ltd., Australia), 2 U Taq polymerase and 14 U AMV RT in a total volume of 25 $\mu$L. Each reaction was overlaid with 60 $\mu$L paraffin oil (BDH Chemicals Ltd., Poole, England) and incubated in a Perkin Elmer Cetus DNA Thermal Cycler according to the following protocol: 60 min at 42°C (to allow reverse transcription), 5 min at 94°C (denaturation of DNA), then 35 cycles of 94°C for 1 min, 60°C for 1 min (primer annealing temperature) and 72°C for 2 min (primer extension), then finally 7 min at 72°C. Conditions that were subsequently altered were the concentrations of Taq polymerase and RT, and the addition of yeast tRNA.

### Agarose gel electrophoresis

Subsequent to PCR, 5 $\mu$l of each PCR product was subjected to electrophoresis through a 1% agarose gel in Tris-borate buffer, stained with ethidium bromide, and viewed and photographed under UV light.

## RESULTS

### Sensitivity of RT-PCR

RRV RNA was added to the RT-PCR reaction in ten fold dilutions from 200 ng to 20 pg of RNA per reaction. As positive controls, 100 pg of plasmid DNA of a cDNA clone of the E2 region were used in two reactions, one with and one without RT. RT-PCR was performed as described, using 14 U AMV-RT and 2 U Taq polymerase. We found that the RT-PCR reactions containing 200 ng and 20 ng of RRV RNA produced the expected 549 bp product, as did the DNA positive control without the RT, but the DNA positive control containing RT did not produce any amplification product. As the only difference between the two positive controls was the addition of RT, this indicated that RT interfered with the amplification of DNA by Taq polymerase.

### Effect of RT on Taq polymerase

In order to determine whether this effect was due to direct action of RT on Taq polymerase, or whether it was due to competition between the two enzymes for vital reaction components, experiments were performed to determine at what stage RT exerted its inhibitory effect.

To examine whether RT affected Taq polymerase directly, 14 U of RT and 2 U of Taq polymerase were incubated for 1 hour at 42°C (simulating their exposure time in the normal reverse transcription step of RT-PCR), and then RT was inactivated by heating the mix to 95° for 5 min. The enzyme mix was then added to the rest of the PCR reaction components (including plasmid DNA as target sequence) and PCR performed. This did not yield any amplification product, indicating that the RT had incapacitated the Taq polymerase.

When the RT was heat inactivated prior to incubation for one hour with Taq polymerase before PCR, the PCR was successful indicating that only non-heat-denatured RT can incapacitate Taq polymerase.

Hence the inhibition of Taq polymerase activity is a direct effect of RT, and this is only exhibited by active RT.

### Ratio of Taq polymerase to RT

To determine whether Taq polymerase was able to tolerate the presence of any RT at all, we then altered the proportions of the two enzymes in the RT-PCR reaction.

Firstly, seven RT-PCR reactions were performed containing 2 U of Taq polymerase, and 5, 4, 3, 2, 1, 0.5 and 0 U of RT respectively, with DNA as target sequence. Reactions with 3 U or less of RT were successful, showing increasing band brightness (and hence product formation and Taq polymerase activity) as the amount of RT decreased (Figure 1).

A set of four similar RT-PCR reactions were performed, using 7 U RT and 2.5, 10, 15 U Taq polymerase respectively. Reactions containing 5 U Taq polymerase or greater were successful, also showing more product formation as Taq polymerase concentration increased.

These reactions show that RT is able to block Taq polymerase activity if the RT:Taq polymerase ratio is greater than approximately 3:2, and the inhibition decreases as RT concentration decreases. This dependence on molar ratios suggests a direct molecular interaction between the two enzymes.

### General effect

The inhibition was shown to be a general effect by substitution of various components of the RT-PCR reaction. Moloney murine leukaemia virus RT (M-MLV RT) was shown to inhibit Taq polymerase when substituted for AMV-RT, hence both RTs can inhibit Taq polymerase. Taq polymerase acquired from a different supplier (Perkin Elmer Cetus : Amplitaq) was also inhibited by both AMV and M-MLV RT. The effect appears not to be sequence restricted, since amplification of a cDNA clone of Murray Valley encephalitis virus (MVE) using MVE specific primers was also inhibited by addition of RT.

### Addition of RNA

In the original experiment, the RT-PCR reactions containing the two highest RRV RNA concentrations were successful. This suggested that if the RNA concentration was high enough, RT

would bind to the RNA rather then interfere with Taq polymerase. To confirm this, four RT-PCR reactions were performed using 14 U RT, 2 U Taq polymerase, DNA as target sequence, and adding 0, 0.2 μg, 2 μg or 20 μg of yeast tRNA respectively. We found that addition of 0.2 μg or greater of yeast tRNA allowed amplification of DNA which would not occur using these levels of RT and Taq polymerase without additional tRNA.

Addition of extra tRNA also increased the sensitivity of detection of viral RNA. By altering RT and Taq polymerase levels to 2 U Taq polymerase and 0.5 U RT we were able to detect a $10^{-5}$ dilution of viral RNA (i.e. 2 pg). By addition of 2 μg yeast tRNA to the reaction, the sensitivity was increased 100 fold (i.e. 20 fg viral RNA detected).

### $MgCl_2$ concentration

To determine whether the inhibition was affected by $MgCl_2$ levels, we performed RT-PCR using $MgCl_2$ concentrations of 2, 3, 4, 5, 6 and 7 mM. This range covered the optimum $MgCl_2$ concentrations for Taq polymerase (2 mM) and RT (6–7 mM). None of these reactions yielded amplification product, hence magnesium levels do not affect the inhibition of Taq polymerase by RT.

### Effect of RT on other reaction components

To determine whether any of the other reaction components were affected by RT, the components were incubated separately with RT for 1 hour at 42°C, then RT was inactivated by Proteinase K digestion followed by heating for 5 min at 95° to inactivate the Proteinase K. The remainder of the components necessary for PCR were then added and PCR was performed. Amplification was successful in all cases, indicating that RT does not inhibit Taq polymerase indirectly by affecting other reaction components, i.e. primers, target sequence, $MgCl_2$, components in the reaction buffer.

When similar reactions were performed using heat inactivation of RT rather than Proteinase K digestion, the PCR was successful if the RT was incubated with the reaction buffer and nucleotides, however no amplification product was obtained if the RT was incubated with primers or target DNA or $MgCl_2$. This indicates that active RT is still present to inhibit the Taq polymerase, and hence that these reactants possibly reduce the susceptibility of RT to heat inactivation. Since each of these components is involved in binding to RT, they may change the enzyme conformation, and hence render it less susceptible to heat inactivation.

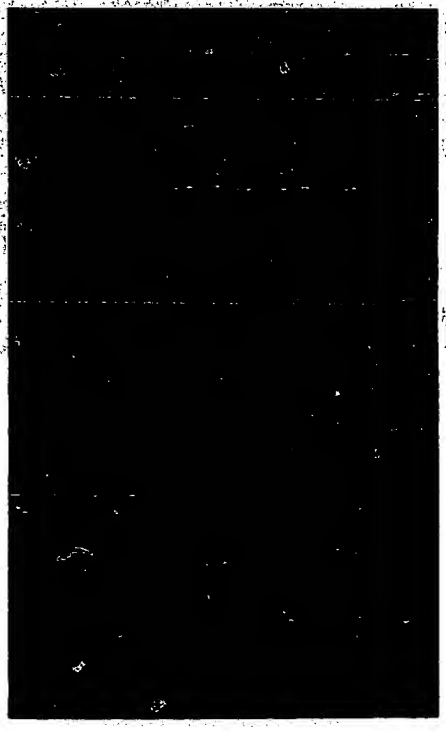

**Figure 1.** RT-PCR of Ross River Virus cDNA. Reaction mixes for lanes 1 to 7 contain 2 units of Taq polymerase each, and 5, 4, 3, 2, 1, 0.5 and 0 units of reverse transcriptase respectively. Lane 8 contains molecular weight marker.

## DISCUSSION

In this paper we have described a systematic investigation which shows that Taq polymerase activity can be inhibited in the presence of reverse transcriptase. This inhibition is caused by a direct action of RT on Taq polymerase.

The degree of Taq polymerase inhibition increases with increasing RT concentration, up to a certain point beyond which Taq polymerase is rendered completely inactive—at a ratio of approximately 3 U RT : 2 U Taq polymerase (for AMV RT). Other researchers may have unwittingly avoided this problem by either using a lower RT : Taq polymerase ratio in RT-PCR, or performing the cDNA synthesis separately and diluting this before adding to the PCR reaction.

This reliance on molar ratios suggests an interaction between the two enzymes at a molecular level, which may take the form of direct binding between the two enzymes. It is interesting to note that while high concentrations of RT will effectively inactivate Taq polymerase, high concentrations of Taq polymerase do not have a noticeable effect on RT activity.

Inactivated RT does not inhibit Taq polymerase, hence protocols in which Taq polymerase is not added until after reverse transcription has taken place and the RT inactivated or removed will not show inhibition of Taq polymerase activity. However once active RT has been exposed to Taq polymerase, the Taq polymerase becomes permanently incapacitated. The inhibition of Taq polymerase cannot be reversed by subsequent heat inactivation of the RT.

The addition of extra non-homologous RNA to the RT-PCR reaction has the effect of decreasing the inhibition of Taq polymerase by RT. This is possibly due to the RNA providing an alternative binding substrate for the RT. Viral RNA that has been prepared by extraction of total RNA from infected cells or tissue will not show such limited sensitivity in an RT-PCR detection system, since extra RNA is already present. Other studies using purified viral RNA in an RT-PCR reaction have been reported which have lower than expected sensitivity (6, 11). Addition of extra non-homologous RNA may improve this.

RT-PCR has in most cases been reported to be 1000-fold or more sensitive than other viral detection systems such as hybridisation, ELISA, or tissue culture. However the theoretically possible detection of one viral genome by RT-PCR remains elusive, and this may in part be due to the inhibition of Taq polymerase by RT. Measures to counter this effect need to be taken especially in situations where the number of genome copies is likely to be very low, such as detection of viral pathogens in

water supplies or sewage. We have found that the optimum RT-PCR conditions for the detection of viral RNA are the use of 0.5 U RT, 2 U Taq polymerase and the inclusion of 2 μg yeast tRNA. This combination allows the ease and practicality of combining reverse transcription and PCR in one reaction tube without interruption, while minimising the inhibitory effect of RT on Taq polymerase.

## ACKNOWLEDGEMENT

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